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Journal

Leukemia, 26(12)

ISSN

0887-6924

Authors

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Publication Date

2012-12-01

DOI

10.1038/leu.2012.143

Peer reviewed



Published in final edited form as:

Leukemia. 2012 December ; 26(12): 2494–2498. doi:10.1038/leu.2012.143.

Leukemia-related chromosomal loss detected in hematopoietic progenitor cells of benzene-exposed workers

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Abstract

Benzene exposure causes acute myeloid leukemia, and hematotoxicity, shown as suppression of mature blood and myeloid progenitor cell numbers. As the leukemia-related aneuploidies monosomy 7 and trisomy 8 previously had been detected in the mature peripheral blood cells of exposed workers, we hypothesized that benzene could cause leukemia through the induction of these aneuploidies in hematopoietic stem and progenitor cells. We measured loss and gain of chromosomes 7 and 8 by fluorescence *in situ* hybridization in interphase colony-forming unit-granulocyte-macrophage (CFU-GM) cells cultured from otherwise healthy benzene-exposed (n=28) and unexposed (n=14) workers. CFU-GM monosomy 7 and 8 levels (but not trisomy) were significantly increased in subjects exposed to benzene overall, compared to levels in the control subjects ($p=0.0055$ and $p=0.0034$, respectively). Levels of monosomy 7 and 8 were significantly increased in subjects exposed to <10 ppm (20%, $p=0.0419$ and 28%, $p=0.0056$, respectively) and 10 ppm (48%, $p=0.0045$ and 32%, $p=0.0354$) benzene, compared with controls, and significant exposure-response trends were detected ($p_{trend}=0.0033$ and 0.0057). These data show that monosomies 7 and 8 are produced in a dose-dependent fashion in the blood progenitor cells of workers exposed to benzene and may be mechanistically relevant biomarkers of early effect for benzene and other leukemogens.

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Conflict of Interest M.T.S. and S.M.R. have received consulting and expert testimony fees from law firms representing both plaintiffs and defendants in cases involving exposure to benzene. G.L. has received funds from the American Petroleum Institute for consulting on benzene-related health research.

Keywords

Benzene; leukemia; monosomy; hematopoietic progenitor

Introduction

Benzene, a widespread environmental pollutant, causes acute myeloid leukemia (AML) and related blood disorders, including myelodysplastic syndromes (MDS)^{1–2}. While the underlying mechanisms are unclear, benzene is a known hematotoxicant³. Previously, we reported reduced peripheral white blood cell and platelet counts in 250 workers exposed to benzene compared with 140 controls, even at levels below the US occupational exposure limit of 1 ppm⁴. In the same study, circulating myeloid progenitor cells, including colony-forming unit–granulocyte-macrophage (CFU-GM), were also found to be significantly reduced in number with increasing benzene exposure and were more sensitive to suppression than mature blood cells⁴. These myeloid progenitor cells circulate in the bloodstream in dynamic equilibrium with the corresponding cell pools in the bone marrow, and are, together with early hematopoietic stem cells, likely targets for induction of myeloid leukemia. Through genotoxic effects, benzene could induce sufficient damage to kill or suppress the proliferation of myeloid progenitor cells. Conversely, a genotoxic effect such as genomic instability that gives cells a survival or proliferation advantage could lead to the generation of pre-leukemic stem or progenitor cells, and induce leukemia.

Aneuploidy is a type of genomic instability that is both implicated in myeloid leukemia and induced by benzene. Two recent studies in yeast⁵ and diverse human cancer cell lines⁶ show that aneuploidy enhances genetic recombination and defective DNA damage repair. These findings provide a mechanistic link between aneuploidy and genomic instability and suggest that aneuploidy can cause a modest mutator phenotype and may be an initiating event in cancer. Monosomy 7 is the most frequent cytogenetic abnormality in therapy-related MDS and AML⁷ and trisomy 8 is also commonly detected in AML⁸. Increased levels of monosomy 7 and trisomy 8 have been detected in the peripheral blood cells of benzene-exposed workers^{9–13} and have also been observed in cultured human lymphocytes and bone marrow cells treated with benzene metabolites^{14–16}. We hypothesized, therefore, that benzene would induce the leukemia-related chromosomal aneuploidies, monosomy 7 and trisomy 8, in the CFU-GM progenitor cells of benzene-exposed workers. We measured loss and gain of chromosomes 7 and 8 in the interphase CFU-GM cells of 28 workers exposed to benzene and 14 controls, by fluorescence *in situ* hybridization (FISH).

Methods

Study population

Study subjects were selected from a cross-sectional study of 250 healthy workers exposed to benzene and 140 unexposed controls and peripheral bloods were collected and processed as described previously^{4, 17}. Briefly, exposed workers were enrolled from two shoe manufacturing factories and unexposed controls were selected from three clothes manufacturing factories in the same region of Tianjin, China. Shoe workers had been

employed for an average of 6.1 ± 2.9 years. Controls were frequency matched by sex and age to exposed workers. The study was approved by Institutional Review Boards at the U.S. National Cancer Institute and the Chinese Academy of Preventive Medicine.

Participation was voluntary, and written informed consent was obtained. For each subject, individual benzene and toluene exposure was monitored repeatedly for up to 16 months before phlebotomy, and postshift urine samples were collected from each subject during the week before phlebotomy and analyzed for benzene by Gas Chromatography with Mass Spectrometry (GC-MS) as previously described¹⁸. The characteristics of the subjects analyzed in the current study, which comprised 42 workers (14 controls, 18 exposed below 10 ppm and 10 exposed at or above 10 ppm benzene), from which at least 100 CFU-GM cells were cultured, are provided in Table 1.

Culturing of myeloid progenitor CFU-GM cells using colony-forming assay

Circulating stem and progenitor cells can be cultured from peripheral blood using colony-forming assays in semi-solid media containing appropriate growth factors. During the culture period, the majority of terminally differentiated cells die out and the progenitor cells establish individual colonies that can be classified microscopically according to the progenitor cell type. While the CFU-GM colony-forming assay is well-established, limitations of the field setting and available personnel make it difficult to perform on a large number of subjects at the time of blood collection. Therefore, as described previously⁴, colonies derived from CD34 positive progenitor cells that give rise to granulocytes and macrophages, called CFU-GM, were cultured from a subset of individuals selected to include controls (n=24) and individuals exposed to a range of benzene exposures (n=29). Briefly, mononuclear cells were isolated from peripheral blood, washed, counted and mixed with growth factor-containing methylcellulose media, MethoCult® H4534 Classic without EPO (Stem Cell Technologies, Inc., Vancouver, Canada). This media contains the growth factors, recombinant human (rh) Stem Cell Factor, rh Granulocyte-Macrophage Colony-Stimulating-Factor, rh Interleukin-3, but does not contain erythropoietin. The cells were plated to a final concentration of 300 000 cells per 1.1 ml petri dish then cultured under strict sterile conditions for 12–14 days at 37°C in a humidified 5% CO₂ incubator. The number of CFU-GM colonies was scored in 6 petri dishes per study subject, by two highly trained personnel (3 dishes each). The average number of CFU-GM colonies per 100 000 plated mononuclear cells was calculated.

Detection of aneuploidy of chromosomes 7 and 8 in CFU-GM cells by FISH

CFU-GM cells in interphase rather than in metaphase were analyzed because the majority of cells are non-dividing allowing a larger number of cells to be scored. At the end of the culture period, CFU-GM cells were harvested from individual colonies, pooled, washed, and dispersed into a hypotonic solution (0.075 M KCl) for 30 min. The cells were fixed in methanol-acetic acid (3:1) twice and then dropped onto several slides, air-dried and stored in slide boxes under nitrogen gas at -20°C. Aneuploidy (monosomy and trisomy) of chromosomes 7 and 8 was examined using a dual-color hybridization by FISH in the interphase cells as previously described¹⁹. Briefly, centromeric chromosome 7 and 8 probes (Vysis®, Abbott Laboratories, Des Plaines, Illinois) were applied to prepared CFU-GM

cells. The probes and target cell DNA were simultaneously denatured, washed rapidly using a formamide-free high-stringency wash solution, and 900 interphase cells on average per subject were evaluated microscopically. As mentioned above, at least 100 CFU-GM cells were available for aneuploidy detection from 14 of the controls and 28 of the exposed samples assayed. The numbers of cells analyzed in each exposure group (mean \pm standard deviations) were as follows: controls (814 ± 535); exposed below 10 ppm (845 ± 415); and, exposed at or above 10 ppm (1023 ± 501). The median number of cells and interquartile range (in parentheses) for each exposure group were as follows: controls (595, 411–1386); exposed below 10 ppm (678, 572–1316); and, exposed at or above 10 ppm (1179, 804–1465).

Statistical analysis

Trends in chromosome 7 and 8 monosomy rates and CFU-GM colony counts with benzene exposure, and differences in monosomy rates and CFU-GM colony counts by benzene exposure category (i.e., unexposed controls vs. workers exposed to <10 ppm and 10 ppm benzene), were tested by fitting appropriate negative binomial regression models. Thus, a log-linear model of mean counts allows for significant over-dispersion, particularly when deriving the statistical inference. Changes in white blood cell (WBC) counts with benzene level, and differences in WBC count by benzene exposure category, were tested by linear regression. All analyses were carried out using SAS 8.0 software (SAS Institute Inc., Cary, North Carolina). Models were adjusted for the potential confounders age and sex, and additionally for smoking, alcohol, recent infections and body mass index (BMI) if $p < 0.05$ (Table 1).

To determine whether there was evidence of a significant departure from log-linearity (e.g. supra-linearity), we used a procedure to consistently estimate the relative ability of a linear model and a completely unspecified smooth model to fit future data via a cross-validation procedure. Specifically, we divided the data into $v=1, \dots, 10$ equal samples (validation samples) and for each chromosome, and each validation sample, we performed the following steps:

1. We fit a log-linear, generalized additive model on the corresponding training sample (that is, -all the data minus the validation sample, v), of the form:

$$\log \{E(Y|A, W)\} = \beta_0 + \beta_1 A + s(W_1) + W_2 + W_3 + W_4 \quad \text{Equation 1}$$

where A is continuous benzene exposure; W_1 is age (with $s(W_1)$ representing a smooth term based upon a reduced rank version of the thin plate splines, which use the thin plate spline penalty, implemented using the *mgcv* library within statistical software, R²⁰); and W_2 , W_3 , and W_4 are binary smoking, alcohol consumption, and presence of infection, respectively. This model was used to predict monosomy on the omitted validation sample, e.g. $v=1$; these predictions were called

$$\hat{p}_{null} \equiv \hat{E}_{null}(Y|A, W)$$

2. We fit the same model, Equation 1, but now with a smooth term for benzene: $s(A)$. We then repeated that prediction for the corresponding validation sample, $v=1$; these modified predictions were called \hat{p}_{alt}
3. Steps 1 and 2 were repeated for all validation samples, $v=1, \dots, 10$, resulting in a data set consisting of two different competing predictions and the outcomes (independent of the data used to make the predictions).
4. Finally, we simply compared the R^2 values, which in this case is simply:

$$R_{null}^2 \equiv \text{cor}^2(Y, \hat{p}_{null}), R_{alt}^2 \equiv \text{cor}^2(Y, \hat{p}_{alt}),$$

Results

Detection of loss, but not gain, of chromosomes 7 and 8

We acquired sufficient CFU-GM cells from 42 subjects (14 controls and 28 workers exposed to benzene) to perform FISH analysis of chromosome 7 and 8 aneuploidy. Monosomy 7 levels (mean \pm SE %, 5.61 \pm 1.68) in the CFU-GM of subjects exposed to benzene overall were significantly increased compared to monosomy 7 levels (4.32 \pm 2.06) in the control subjects ($p=0.0055$). Similarly, monosomy 8 levels (6.75 \pm 1.83) were significantly increased compared to values (5.21 \pm 2.17) in the control subjects ($p=0.0034$). No significant change in trisomy 7 or 8 with overall benzene exposure was detected.

Dose-dependent increase in monosomy 7 and 8

The benzene exposed subjects were divided into two exposure groups, below 10 ppm ($n=18$) and at or above 10 ppm ($n=10$), in order to evaluate dose-response effects on monosomies 7 and 8. At <10 ppm benzene, levels of monosomy 7 and 8 in CFU-GM were significantly increased by 20% ($p=0.0419$) and 28% ($p=0.0056$), respectively, compared with controls. At 10 ppm, levels of monosomy 7 and 8 in CFU-GM were significantly increased to a greater degree, by 48% ($p=0.0045$) and 32% ($p=0.0354$), respectively, compared with controls. The exposure-response trends in monosomy 7 and 8 with increasing exposure were highly significant ($p_{trend}=0.0033$ and 0.0057, respectively), Figure 1.

We used a cross-validation procedure (10-fold) to examine whether there was a significant departure from log-linearity in the dose-response. For each of the training samples, we estimated two models of continuous benzene exposure versus mean monosomy rate: a linear model and a completely unspecified smooth model (generalized additive model; GAM²¹). For each training sample, we predicted on the corresponding validation sample, so that we derived an unbiased estimate of the fit of the two approaches. The models revealed nearly identical fits, with $R_{null}^2=0.60$, $R_{alt}^2=0.59$ for monosomy 7 and $R_{null}^2=0.40$, $R_{alt}^2=0.38$ for monosomy 8 (Figure 2). Though the increase in flexibility of these models reduced the bias of a smaller model, it resulted in added variance. These data do not provide any evidence of a departure from a log-linear dose-response in monosomy 7 and 8 versus benzene exposure. Thus, increasing exposure to benzene appears to induce monosomy 7 and 8 in a linear fashion.

Comparison of increased monosomies with decreased WBC counts

WBC and CFU-GM colony counts were significantly lowered in the group of workers exposed to 10 ppm ($p < 0.01$), but not in the group exposed to <10 ppm, relative to controls (Figure 3). However, the inverse trends for both WBC and CFU-GM colony counts with increasing exposure were highly significant ($p_{\text{trends}} = 0.0027$ and 0.0007 , respectively). In both the <10 ppm and 10 ppm exposure groups, the proportional increase in monosomy 7 in the myeloid progenitor cells relative to controls (20% and 48%, respectively) was greater than the corresponding decreases in both WBC (5% and 25%) and CFU-GM (5% and 30%) counts. Similarly, in both the <10 ppm and 10 ppm exposure groups, the proportional increases in CFU-GM monosomy 8 levels relative to controls (28% and 32%, respectively) were greater than the corresponding decreases in WBC (5% and 25%). The increase in CFU-GM monosomy 8 level (28%) was also proportionally greater than the decrease in CFU-GM (5%) count at <10 ppm benzene.

Discussion

Previously, we reported the detection of increased levels of the leukemia-related aneuploidies monosomy 7 and trisomy 8 in the mature peripheral blood cells of benzene-exposed workers⁹⁻¹³. We also reported that early myeloid progenitor (CFU-GM) cells, possible targets for leukemogenesis, are more sensitive than are mature cells to the hematotoxic effects of benzene in exposed workers⁴. In the present study, we found that benzene exposure was associated with increased levels of monosomy 7 and 8 in the CFU-GM cells from a subset of individuals in the hematotoxicity study. This association is apparent in the ~30% increase in both monosomy 7 and 8 observed in the benzene-exposed group overall compared with the unexposed controls, and the even greater increases (48% and 32%, respectively) observed in the group exposed to 10 ppm benzene. Thus, we found effects above and below 10 ppm, which was the voluntary industrial exposure limit adopted by OSHA from 1971 to 1977.

The apparent lack of an increase in trisomy was surprising given that we previously reported the detection of increased levels of trisomy, as well as monosomy, in the peripheral blood lymphocytes of healthy workers exposed to high levels of benzene^{9, 10, 13, 22-23} and to a range of benzene levels¹¹. The background rate of trisomy in peripheral blood lymphocytes was much lower than that of monosomy in the previous studies and is also lower in hematopoietic progenitors in the current study. This, together with the small number of subjects and cells examined in the current study and the reduced sensitivity of interphase FISH to detect trisomy compared with metaphase FISH¹⁹, may have reduced the ability to detect an increase in trisomy rate.

It seems unlikely that the chromosomal changes we observed were due to confounding factors. All the workplaces studied were in the same geographic region and the enrolled workers had comparable demographic and socioeconomic characteristics and were engaged primarily in manufacturing. Thus, significantly different dietary, genetic, and environmental factors, other than benzene, are unlikely to explain the results. In addition, models were adjusted for the potential confounders, age and gender, and additionally for smoking, alcohol, recent infections and body mass index.

The mechanism by which benzene induces monosomy 7 and other aneuploidies is unknown. Aneuploidy in somatic cells has been associated with defects in mitotic spindle assembly and dynamics, centrosome amplifications, cell-cycle regulation, chromatid cohesion, and telomere metabolism. These defects may be caused by mutation or altered expression of the genes involved in these processes. Several benzene metabolites including semiquinone radicals and quinones, in particular benzoquinone (BQ), produced by peroxidases in the bone marrow, are highly toxic by directly binding to cellular macromolecules and generating oxygen radicals through redox cycling^{24–25}. In particular, benzene metabolites have been shown to disrupt microtubules, by targeting tubulin^{26–28}, representing a possible mechanism of aneuploidy induction. DNA hypomethylation of sub-telomeric, telomeric, and pericentric regions has also been implicated in aneuploidy induction²⁹. We have previously reported that hydroquinone causes global DNA hypomethylation *in vitro*³⁰. Recently, HQ was shown to induce aneuploidy in yeast through the HOG1-SWE1 pathway, which controls p38MAPK-dependent G2 phase to M phase transition³¹. HQ treatment caused the phosphorylation of Hog1, but not Rad53, followed by the accumulation and stabilization of Swe1, leading to the inhibition of mitosis and a delay in the cell cycle at the G2/M transition. Each of these potential mechanisms requires further investigation.

One potential criticism of our study is the relatively high number of monosomy 7 and 8 events observed in the control cells (mean±SE %, 4.32±2.06 and 5.21±2.17, respectively). This is likely due to a well-known artifact produced by probe overlap in the interphase FISH assay¹⁹, rather than by the presence of monosomy 7 in the control individuals. We chose interphase FISH over metaphase FISH to maximize the number of progenitor cells that could be examined, thus increasing the power to detect true differences in levels between exposed and unexposed individuals. Probe overlap due to apoptotic condensation could also potentially result in an elevated number of nuclei with one signal, as was previously suggested¹⁴. Despite the relatively high background, which is actually lower than was reported previously¹⁹, we found highly significant differences between controls and benzene-exposed individuals.

We conclude that occupational exposure to benzene induces monosomy 7 and 8 in the myeloid progenitor cells of otherwise healthy workers, providing a mechanistic basis for leukemia induction by benzene. Although induction of these monosomies was previously reported in the lymphocytes of benzene-exposed workers, its detection in multipotent hematopoietic progenitors in the present study further strengthens it as a potential mechanism. Further, the degree of monosomy induction was greater than the proportionate decline in peripheral blood cell counts, suggesting that it may be a more sensitive biomarker of benzene exposure. Future studies should validate this finding in a separate benzene-exposed population; examine potential susceptibility factors; and conduct *in vitro* studies to determine the mechanisms underlying the induction of monosomy and the roles of individual benzene metabolites.

Acknowledgments

We thank the participants for taking part in this study. Supported by NIH grants RO1ES06721, P42ES04705, and P30ES01896 (MTS), P42ES05948 and P30ES10126 (SMR), and NIH contract N01-CO-12400 with SAIC-Frederick, Inc.

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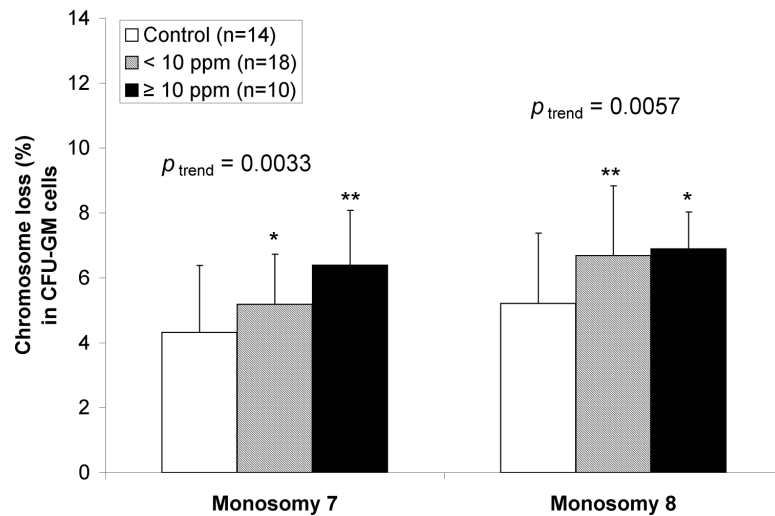


Figure 1. Monosomy 7 and 8 in CFU-GM cells of unexposed controls and subjects exposed to <10 ppm and 10 ppm benzene

Trends in chromosomal monosomy rates with benzene exposure and differences in monosomy rates by benzene exposure category (i.e., unexposed controls vs. workers exposed to <10 ppm and 10 ppm benzene) were tested by fitting appropriate negative binomial regression models. Models were adjusted for age and sex, and additionally for smoking, alcohol, recent infections and BMI if significant (Table 1). Significant p_{trend} values are shown. p values are indicated as * $p < 0.05$; ** $p < 0.01$. Levels of monosomy 7 and 8 in CFU-GM were significantly increased at <10 ppm and 10 ppm benzene and with increasing benzene exposure

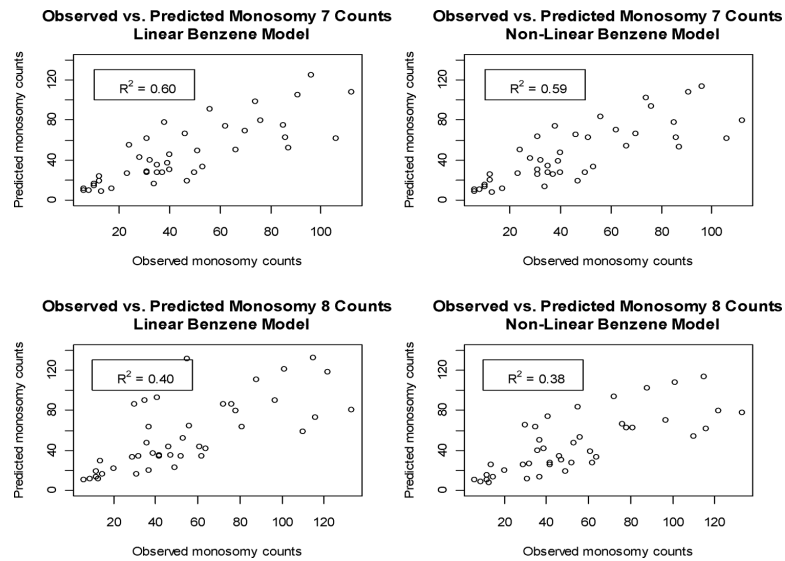


Figure 2. Linear vs non-linear models of monosomy 7 and 8 versus benzene exposure
 Observed versus predicted monosomy 7 and 8 counts versus benzene exposure, determined using a cross-validation approach in linear and non-linear (completely unspecified smooths) models, are plotted. There is no significant difference in the relative fit of the models.

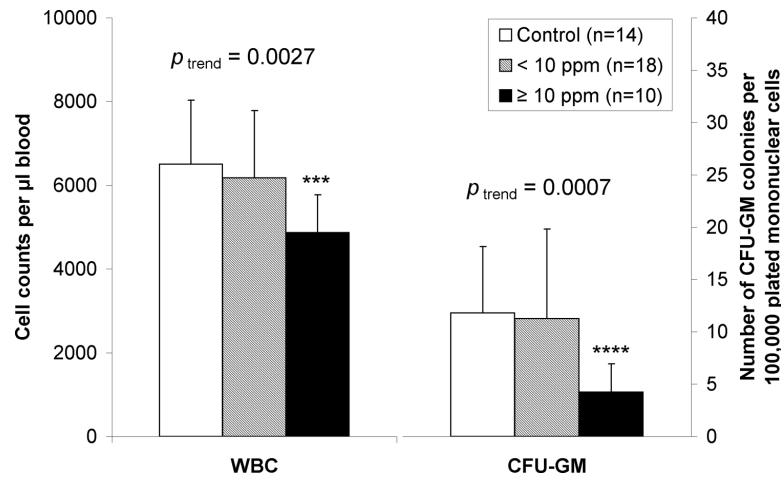


Figure 3. White blood cell (WBC) count and number of CFU-GM colonies in unexposed controls and subjects exposed to <10 ppm and ≥ 10 ppm benzene

Trends in WBC cell or CFU-GM colony counts with benzene level, and differences in cell or colony count by benzene exposure category, were tested by negative binomial regression (CFU-GM) and linear regression (WBC). Models were adjusted for age and sex, and additionally for smoking, alcohol, recent infections and BMI if significant (Table 1). Significant p_{trend} values are shown. p values are indicated as *** $p < 0.001$, and **** $p < 0.0001$. WBC and CFU-GM colony counts were significantly lowered in the ≥ 10 ppm exposed group of workers and with increasing benzene exposure.

Table 1

Demographic and exposure characteristics of study subjects from Tianjin, China

| Benzene Exposure | Subject (n) | Benzene Air Level* (ppm) | Benzene Urine Level (µg/L) | Gender | | Age (Year) | | Currently Smoking | | Currently Drinking Alcohol | | Recent Respiratory Infections | | Body Mass Index |
|------------------|-------------|--------------------------|----------------------------|----------|--------|------------|--------|-------------------|--------|----------------------------|---------|-------------------------------|----|-----------------|
| | | | | Male | Female | Yes | No | Yes | No | Yes | No | Yes | No | |
| Controls | 14 | 0.04±0.00** | 0.25±0.61 | 5(36)*** | 9(64) | 3(21) | 11(79) | 5(36) | 9(64) | 2(14) | 12(86) | | | 22.28±3.36 |
| <10 ppm | 18 | 2.64±2.70 | 66.39±138.49 | 2(11) | 16(89) | 2(11) | 16(89) | 1(6) | 17(94) | 0(0) | 18(100) | | | 21.22±2.76 |
| 10 ppm | 10 | 24.19±10.64 | 897.70±874.63 | 6(60) | 4(40) | 5(50) | 5(50) | 7(70) | 3(30) | 3(30) | 7(70) | | | 22.21±2.38 |

* Air sample collected one month before blood collection;

** Data present mean values ± SD;

*** Subject N (%)